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# Diagnostic Assessment of an Enzyme-Linked Immunosorbent Assay for Human and Canine Blastomycosis

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**An enzyme-linked immunosorbent assay (ELISA) for blastomycosis was evaluated with 65 human sera from culturally or histologically proven cases of blastomycosis, 53 sera from humans with heterologous infections, and 115 sera from apparently normal people. The diagnostic value of the ELISA was compared with that of the widely used complement fixation (CF) and immunodiffusion (ID) tests. The assay gave a sensitivity of 80% and a specificity of 98% with a minimal positive titer of 1:16. In contrast, the CF and ID tests demonstrated sensitivities of only 40 and 65%, respectively, and both were 100% specific. It was concluded that ELISA titers of 1:32 or greater are indicative of active blastomycosis, whereas lower titers, which might represent cross-reactions, were considered suggestive of the disease. The specificity of low titers should be confirmed by immunodiffusion tests or from the study of serial serum specimens. Preliminary studies with sera from 6 dogs with active blastomycosis and 31 asymptomatic dogs revealed an ELISA sensitivity of 100% and a specificity of 97% when a 1:8 cutoff titer was used. Although a wide range of titers was obtained in both human and canine specimens, no single titer could be relied on to reflect the clinical form of disease. However, a four-fold-or-greater reduction in titer for serial specimens appeared indicative of a favorable prognosis.**

The clinical diagnosis of blastomycosis has long been a challenge to clinicians and laboratorians. The symptoms of blastomycosis are nonspecific and frequently mimic other types of respiratory illness (12). Isolation and identification of *Blastomyces dermatitidis* provides the most definitive proof of blastomycosis. However, this approach can be time consuming and at times may yield false-negative results. Histologic studies may also enable an early and accurate diagnosis, but this approach may not always be taken due to the need for invasive procedures. Serologic tests, in general, provide diagnosis faster than cultural and histologic techniques, but until recently, tests applied to the diagnosis of blastomycosis have been plagued with low sensitivity and nonspecificity.

Until the introduction of the specific *B. dermatitidis* A antigen in the immunodiffusion (ID) test in 1973 (5), the most widely used serodiagnostic test for blastomycosis was the complement fixation (CF) with a yeast-form antigen. The latter test, however, was low in both sensitivity (57%) and specificity (30%). Sensitivity and specificity were greatly enhanced with immunodiffusion tests with culture filtrates containing the A antigen and reference sera with A precipitin (2, 14). Subsequently, purified A antigen was isolated and used in CF tests instead of crude yeast antigen (3). This resulted in a CF test that was specific but had a sensitivity of only 62%. A promising enzyme immunoassay in which the A antigen was used was reported at the same time to have a sensitivity of 92% and a specificity of 84%. Unfortunately, the number of specimens used in that preliminary report was limited.

The purpose of this study was to investigate the serodiagnostic value of purified A antigen in an enzyme-linked immunosorbent assay (ELISA) for blastomycosis and to compare its diagnostic efficacy with that of the CF and ID tests. The applicability of the assay for diagnosing canine blastomycosis was also investigated.

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## MATERIALS AND METHODS

**Preparation of antigen.** The A antigen used in these studies was purified by modification of the method described by Green et al. (3). *B. dermatitidis* CDC X-79 yeast-form cells were grown in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) at 37°C for 7 days (5). The cells were killed with merthiolate at a final concentration of 0.02%, and the suspension was passed through a filter (pore size, 0.45 μm). The yeast cells were retained, and the culture filtrate was concentated 10-fold and dialyzed extensively with 0.20 M Tris buffer (Tris hydrochloride; pH 8.6; Fisher Scientific Co., Pittsburg, Pa.). The yeast cells were extracted as follows. The yeast cells were suspended in 0.02 M phosphate-buffered saline (PBS; pH 7.6) at a concentration of 1% (vol/vol). The suspension was incubated at room temperature for 2 weeks and shaken manually two to three times a day. The cells were filtered, and the filtrate (PBS-extract) was concentrated 10-fold and dialyzed with Tris hydrochloride buffer. Twenty-five milliliters of the 10-fold concentrated culture filtrate or of the PBS-extract was applied to a DEAE-Sepharose CL-6B ion-exchange column (2.5 by 20 cm; Pharmacia, Uppsala, Sweden). After the column was washed with two column volumes (150 ml) of Tris hydrochloride buffer, the column was eluted with a linear gradient produced from equal volumes (150 ml each) of 0.02 M Tris hydrochloride and 0.02 M Tris–1 M NaCl (pH 8.6) buffers. The fractions were assessed for the A antigen by ID (6) with rabbit anti-*B. dermatitidis* A serum (2). The A antigen containing fractions were dialyzed against 0.02 M Tris hydrochloride buffer and reapplied to the DEAE column that had been reequilibrated with 10 column volumes of Tris buffer. The column was eluted as previously described. The A-antigen-containing fractions were pooled, dialyzed, and adjusted to a concentration of 15 μg of protein per ml (7).

**Serum specimens.** Serum specimens were obtained from patients with histologically or culturally proven (or both) blastomycosis (65 cases), coccidioidomycosis (12 cases), cryptococcosis (3 cases), histoplasmosis capsulati (24 cases), paracoccidioidomycosis (2 cases), and sporotrichosis

(5 cases). Sera from four patients with diabetes and from three patients with respiratory illness who were serologically positive for *Mycoplasma pneumoniae* were also tested. Sera from 6 dogs with culturally or histologically proven blastomycosis and sera from 31 normal asymptomatic dogs were evaluated in the ELISA for canine blastomycosis. The human and canine specimens were obtained after routine diagnostic evaluation by the Fungus Immunology Branch, Division of Mycotic Diseases, Center for Infectious Diseases, or were donated by Carl Armstrong, Virginia Department of Health; Stanley Chapman, Mississippi Veterans Administration; Bruce Klein, Department of Medicine, University of Wisconsin, Madison; or Gene Scalarone, Department of Microbiology, Idaho State University, Pocatello. One hundred fifteen human sera from apparently normal individuals, most of whom reside in the Atlanta area, were also studied.

**Serologic methods.** ID tests with culture filtrate antigen and CF tests with purified A antigen were performed as previously described (6). Radial ID assays were performed as described (8) with glycine-buffered ID agar (6). The ELISA used was a modification of a standard indirect enzyme immunoassay (13). Purified A antigen, optimally diluted (77 ng/ml) in 0.05 M sodium carbonate (pH 9.6) buffer, was adsorbed overnight at 4°C in Immulon II (Dynatech Laboratories, Inc., Alexandria, Va.) ELISA plate wells. The plate was washed with 0.05 M PBS containing 0.05% Tween 20 (Fisher Scientific). Serum specimens (human or canine) were diluted 1:8 in PBS-Tween 20 containing 0.1% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.). Twofold serial dilutions were performed in the wells of the plate with this buffer. The plate was incubated for 30 min in a 27°C water bath and then washed with PBS-Tween 20. Two hundred microliters of horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (heavy and light chain specific) or anti-dog IgG (heavy and light-chain specific) (Cooper Biomedical, Inc., West Chester, Pa.) optimally diluted in PBS-Tween 20-bovine serum albumin was added to each well, and the wells were incubated for 30 min in a 27°C water bath. The wells were washed with PBS-Tween 20. Two hundred microliters of 0.003% $H_2O_2$ (Malinckrodt, Inc., St. Louis, Mo.) containing 0.01% (wt/vol) orthophenylenediamine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added, and the wells incubated in the dark for 30 min at room temperature. The reaction was stopped with the addition of 50 μl of 8 N $H_2SO_4$ (Malinckrodt). $A_{490}$ was measured on an MR 600 reader (Dynatech) after zeroing against pooled normal human or canine sera diluted 1:16. Any absorbance value greater than or equal to 0.200 was considered positive. The highest dilution which gave a positive result was considered the titer.

**Statistics.** Analyses were done to determine sensitivity, specificity, and positive and negative predictive values (11). $\chi^2$ values for sensitivity and specificity were determined by comparing the results of only those specimens which were positive by one test and negative by the other (1). The specimens that gave identical results (positive by both or negative by both) were not used in the $\chi^2$ analysis. Sera with low, medium, or high titers were tested in triplicate three times a week for 2 weeks by the ELISA. The mean and standard deviation of the mean were determined for the cutoff absorbance values.

## RESULTS

**Antigen production.** *B. dermatitidis* CDC X-79 was evaluated for its ability to produce the A antigen. The concentrated culture filtrate and cellular PBS-extract were subjected to analysis in ID and radial ID tests. The culture filtrate contained almost twice the amount of A antigen that the PBS-extract contained. Approximately 500 μg of protein was obtained from each 25 ml of concentrated culture filtrate that was applied to the ion-exchange column. The purified A antigen was stored in 0.02 M Tris hydrochloride buffer at −20°C for at least 12 months with no noticeable loss in activity.

TABLE 1. Diagnostic value of the blastomycosis ELISA with 1:8 and 1:16 minimal titers for sera of apparently healthy subjects and patients with blastomycosis and other diseases

| Minimal titer | Predictive value of a positive reaction (%) | Predictive value of a negative reaction (%) | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|
| 1:8 | 85 | 95 | 86 | 92 |
| 1:16 | 94 | 93 | 80 | 98 |

**Evaluation of minimal positive titer.** The indirect ELISA with purified A antigen was evaluated by using 1:8 and 1:16 as the minimal significant titers for diagnosis of disease (Table 1). A test sensitivity of 86% was noted when a minimal titer of 1:8 was used. An additional four sera were negative when a minimal serum titer of 1:16 was used. This caused an additional 6% drop in sensitivity. Statistical evaluation indicated that this difference was not significant ($\chi^2$ = 2.2). A wide range of titers was observed with the sera from patients with blastomycosis. Approximately, 60% of the patients had acute pulmonary blastomycosis and showed ELISA titers ranging from 0 to 1:1,024. Twenty percent of the patients had either chronic pulmonary or disseminated infection. Dissemination involved bone, skin, meninges, or multiple organs. The sera from these patients demonstrated ELISA titers ranging from 1:16 to 1:2,048. Clinical data on the remaining patients with blastomycosis were not available, but sera from these patients demonstrated titers similar to those seen with the clinically defined cases. Ten of the 46 sera from patients with heterologous mycoses (one patient with coccidioidomycosis, two with cryptococcosis, three histoplasmosis capsulati, four sporotrichosis) and 3 sera from normal people cross-reacted with a 1:8 minimal titer, whereas only 3 sera from patients with mycoses (two patients with histoplasmosis capsulati and one with sporotrichosis) cross-reacted when a 1:16 minimal titer was used. The difference between the specificities of the two tests was found to be significant ($\chi^2 \doteq 8.1$). The two histoplasmosis case sera which cross-reacted at 1:16 were from acutely and chronically ill patients, whereas the third specimen with the 1:8 titer was from a patient with disseminated histoplasmosis. It was evident from the data that the predictive value of a positive reaction increased 9% when a 1:16 cutoff titer was used as compared with the value obtained with a 1:8 cutoff titer. The predictive value of a negative reaction correspondingly dropped only 2%.

The data that follow were based on a test with an initial serum dilution of 1:8 and a minimal positive titer of 1:16. Statistical analysis of the cutoff absorbance value gave a mean of 0.33 (standard deviation, 0.06) with acceptable limits of 0.201 to 0.460. The acceptable limits of a cutoff titer were found to be plus or minus one twofold dilution at a 95% confidence level.

**Comparison of ELISA and ID and CF tests.** The ELISA

and ID and CF tests were compared for their value in the serodiagnosis of blastomycosis. Both the ID and the CF tests were 100% specific for blastomycosis (Table 2). In contrast, sera from two patients with histoplasmosis capsulati and one patient with sporotrichosis cross-reacted in the ELISA, giving a specificity of 98%. These cross-reactions occurred at a 1:16 titer. No cross-reactions occurred at a titer of 1:32 or greater. Statistical evaluation revealed no significant difference in specificity for the ELISA versus the ID or CF test ($\chi^2 = 1.3$).

The ELISA proved to be much more sensitive than either the ID ($\chi^2 = 6.7$) or CF ($\chi^2 = 25.3$) test. Of all the tests, the CF test had the lowest sensitivity since it detected only 26 of the 65 blastomycosis cases. The ID test detected 42 of the 65 cases. The ELISA was positive with sera from 52 of the 65 blastomycosis patients, with titers from 1:16 to 1:2,048. Three of the 13 case specimens missed by the ELISA were positive in the ID test. Thus, blastomycosis can be predicted with an 85% sensitivity and a 98% specificity by performing both the ELISA and the ID tests simultaneously.

**Prognostic value of ELISA.** Serial serum specimens from five patients with acute pulmonary blastomycosis were tested by ELISA to determine the prognostic value of the assay (Table 3). The acute-phase specimens were obtained before treatment except for those from patients 2 and 5, who received therapy approximately 2 weeks before the sera were obtained. The remaining patients (patients 1, 3, and 4) were placed on therapy 1 month after the acute-phase specimens were drawn. The early- and late-convalescent-phase specimens were obtained 4 and 8 months, respectively, after the acute-phase specimen. The titers of two of the patients dropped significantly during early convalescence (patients 3 and 4). All five patients showed fourfold-or-greater declines in ELISA titers during late convalescence. All of the patients were declared clinically cured when the late-convalescent-phase specimens were obtained, and three of the five late convalescent-phase specimens (patients 1, 3, and 5) had a titer below the minimal positive titer of 1:16.

**Diagnostic and prognostic values of ELISA in canine blastomycosis.** A 1:8 titer was used as a minimal positive reaction in the ELISA for canine blastomycosis. Only one of the 31 sera from normal subjects gave a positive reaction at this titer. These tests showed a specificity of 97%, a predictive value of 83% for a positive reaction, and a predictive value of 100% for a negative reaction. All of the six canine sera that were proven to be positive for blastomycosis were positive with titers from 1:8 to 1:512, indicating an excellent test sensitivity (100%). Titers greater than 1:8 were seen in 83% of the sera from proven cases of blastomycosis.

The prognostic value of the ELISA was evaluated on serial serum specimens obtained at 3-month intervals from two dogs. One of the treated dogs became asymptomatic, and his ELISA titer became negative. The serum from this dog demonstrated an ELISA titer of 1:32 before treatment. The other dog similarly became asymptomatic and the serum from this dog demonstrated an eightfold decline in titer, from 1:128 to 1:16. This serum was initially sent as a negative control, but it demonstrated a 1:128 titer and an A precipitin for blastomycosis.

TABLE 2. Comparative values of ELISA and ID and CF tests for serodiagnosis of blastomycosis

| Test | Predictive value of a positive reaction (%) | Predictive value of a negative reaction (%) | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|
| ELISA | 94 | 93 | 80 | 98 |
| ID | 100 | 88 | 65 | 100 |
| CF | 100 | 81 | 40 | 100 |
| ELISA and ID | 95 | 94 | 85 | 98 |

TABLE 3. Prognostic value of ELISA titers with acute- and convalescent-phase sera from five patients with acute pulmonary blastomycosis

| Patient | ELISA titer of: | | |
|---|---|---|---|
| | Acute phase specimen | Convalescent-phase specimen | |
| | | Early phase | Late phase |
| 1 | 32 | 16[a] | 8[a] |
| 2 | 128 | 128 | 16[a] |
| 3 | 256 | 32 | 8[a] |
| 4 | 256 | 64 | 16[a] |
| 5 | 32 | 16 | 0[a] |

[a] Patient was clinically cured at this point.

## DISCUSSION

A reliable and sensitive quantitative serologic test for diagnosing blastomycosis has long been needed (9, 10, 12). The ELISA with purified A antigen proved to be a positive step toward the development of such a procedure. A relatively sensitive (80%) and highly specific (98%) test was achieved by using a minimal 1:16 titer for predicting the disease (Tables 1 and 2). Nonspecific reactions, although minimized, occurred with 6% of 53 sera from patients with documented heterologous diseases including 8% of 24 sera from patients with histoplasmosis capsulati. None of the 115 normal sera were positive at the 1:16 titer, and none of the heterologous sera demonstrated reactivity beyond the 1:16 dilution.

A minimum 1:8 titer has been used in previous ELISAs for blastomycosis (3; B. J. Klein, J. N. Kuritsky, W. A. Chappell, L. Kaufman, J. Green, S. F. Davies, J. E. Williams, and G. A. Sarosi, Am. Rev. Respir. Dis., in press). Our statistical studies revealed that sensitivity was not significantly increased by using a 1:8 titer for a minimal positive reaction but specificity was profoundly affected. Significantly more control sera (8%), including 2.6% of those from apparently normal individuals, cross-reacted when a 1:8 cutoff titer was used. Thirteen percent of the sera from patients with histoplasmosis cross-reacted with a 1:8 minimal cutoff titer. These results are in close agreement with those reported by Green et al. (3) (84% specificity with 12 sera from patients with histoplasmosis) but not with those reported by Klein et al. (in press), who noted 50% cross-reactivity with six sera from patients with histoplasmosis. The unusual cross-reactivity noted by Klein et al. may have been due to differences in the number and type of specimens positive for histoplasmosis.

Ten of the 13 blastomycosis cases missed by ELISA when a 1:16 cutoff titer was used were not positive by any serologic test. The remaining three cases missed by ELISA were positive in the ID test. Why the more sensitive ELISA test did not detect antibody in these specimens when the ID test did is not readily understood. At least two possibilites exist: (i) the antibody produced by these patients was not of a class reactive with the conjugated serum, or (ii) an antigenic component of *B. dermatitidis* culture filtrate (used in

the ID test) was removed during the preparation of the purified A antigen or was incapable of binding to the ELISA plate under the conditions used. For the latter possibility, it is assumed that *B. dermatitidis* produces more than one antigenic component. This is consistent with the report by Young and Larsh (15), who demonstrated, by polyacrylamide gel electrophoresis, that the A antigen contained numerous proteins and glycoproteins, two of which appeared to be associated with antigenic activity.

The serologic results indicate that diagnosis could best be accomplished by concurrent performance of both ELISA and ID tests (Table 2). Accordingly, it is suggested that the following format be followed for diagnosing blastomycosis in humans. Sera from patients suspected of having blastomycosis can be tested by the ELISA and the ID test for antibody to the A antigen of *B. dermatitidis*. An A band in the ID test is considered diagnostic for blastomycosis. ELISA titers equal to or greater than 1:32 are considered indicative of active blastomycosis in persons with no previous history of the disease regardless of the ID result. ELISA titers of 1:8 to 1:16 are regarded as suggestive of blastomycosis. Although patients with blastomycosis may show such titers (approximately 17% of those studied), it should be kept in mind that cross-reactivity at these low levels may also occur with sera from patients with coccidioidomycosis, cryptococcosis, histoplasmosis, and sporotrichosis and with sera from apparently normal persons. Consequently, a diagnosis of blastomycosis must be confirmed either by demonstration of A precipitins in ID or by the study of follow-up specimens to demonstrate ELISA titers of 1:32 or both.

The ELISA appears to be useful for monitoring patients with blastomycosis. Although the number of cases studied was limited, a fourfold-or-greater decline in titer or a reduction to a 1:16 or lower titer offered evidence of a favorable prognosis (Table 3). A reduction to a 1:8 titer might indicate when therapy could be discontinued. Further studies are required to determine the clinical significance of 1:8 and 1:16 titers in treated patients.

The availability of an accurate and fast diagnostic test for canine blastomycosis is very important since the disease affects many canines and progresses rapidly with fatal consequences in the majority of cases (4). Preliminary studies on the applicability of the ELISA for diagnosing canine blastomycosis showed that the assay provided excellent sensitivity and specificity with the sera tested from dogs with blastomycosis. One of the 31 normal dog sera demonstrated a 1:8 titer. This, however, may not have represented a nonspecific reaction since all of the sera tested came from dogs residing in a area endemic for blastomycosis. Consequently, this dog may have had asymptomatic blastomycosis. One of the canine serum specimens from an apparently normal dog, which was initially sent as a negative control, had both an ELISA titer of 1:128 and a precipitin to the A antigen. The dog from whom the serum was taken subsequently became ill, suggesting that the ELISA may be able to detect blastomycosis in dogs before obvious clinical signs appear.

In dogs, as in human blastomycosis, a fourfold-or-greater decline in ELISA titers with serial serum specimens is suggestive of a favorable prognosis. However, the clinical signs of the canine patients must be thoroughly evaluated, as must those of human patients, to ascertain how long treatment should continue. To our knowledge, this is the first report of the use of an ELISA for detecting canine blastomycosis. More extensive studies with additional canine blastomycosis and heterologous sera must be done to prove the usefulness of this serologic test.

ELISA antibody titers ranging from 1:8 to 1:2,048 have been detected in human patients and titers from 1:8 to 1:512 in canine patients. Although titers of 1:32 or greater are accurate indicators of human blastomycosis, no single titer could be relied upon to indicate the clinical form of the disease. The rapidity and accuracy of the ELISA exceeds that of the conventional serologic tests and justifies its use in clinical diagnostic laboratories. Characteristics of the antibody and of the antigen of *B. dermatitidis* are both currently under investigation. Additional studies are in progress to further improve the sensitivity and specificity of the ELISA by modification of the current test or by further purification of the A antigen.

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